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ACTION OF PANCREATIC LIPASE ON MONOMERIC TRIPROPIONIN IN THE PRESENCE OF WATER-MISCIBLE ORGANIC COMPOUNDS

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SUMMARY

The addition of low concentrations of some water-miscible organic compounds (dioxane, acetonitrile, *tert.*-butanol, formamide) to an aqueous system induces a substantial increase of the activity of pancreatic lipase (EC 3.1.1.3) towards monomeric tripropionin. This effect is due to a rise of the maximum velocity (V) of the reaction. K_m becomes less favorable upon solvent addition with the result that, for all substrate concentrations lower than the saturating value, lipase activity passes by a maximum in a relatively narrow solvent concentration range.

The fact that lipase is able to attack tripropionin monomers in organic solvent–water systems is confirmed by the complete disparation of the triglyceride after a 40% hydrolysis. The main product of the reaction is dipropionin.

The finding that lipase can display a strong activity in the complete absence of substrate aggregates and interfaces is discussed in the light of current concepts concerning the mechanism of action of this enzyme. Hydrophobic interfaces and low concentrations of organic solvents have been reported to modify the structure of liquid water. The assumption is made that the modification favors the action of lipase on tripropionin monomers through a transconformation of the enzyme molecule or any other process.

INTRODUCTION

The most characteristic property of pancreatic lipase (triacylglycerol lipase, EC 3.1.1.3) and probably of other lipolytic enzymes is their comparatively weak activity towards fully dispersed substrate molecules in an aqueous system and the considerable activity increase that occurs when the substrate is presented in the form of aggregates (emulsified particles [1] or micelles [2]) separated from water by an interface. Several hypotheses have so far been formulated to explain this unusual property: (a) Native lipase in an aqueous solution is inactive or almost inactive, and its efficiency as an enzyme is very much increased by a limited conformational change resulting from adsorption at an hydrophobic interface [3]. (b) Lipase should be a weaker nucleophile than ordinary esterases and it is unable for this reason [4] to penetrate the organized water shell reported to exist around dissolved hydrophilic

molecules. (c) By analogy with pancreatic phospholipase A_2, lipase may be assumed to possess a special site (induced by interfaces) designated penetration or more simply recognition site (ref. 5 and de Haas, G. H., personal communication) conferring to the enzyme a high reactivity towards interfaces. (d) Lipase finds at an interface a higher substrate concentration than in the liquid mass [6] or a better orientation of the substrate molecules with respect to the geometry of its active site [7].

The present report will show that the rate at which monomeric tripropionin can be attacked by lipase is substantially increased by addition to the system of low concentrations of various water-miscible organic compounds such as acetonitrile, dioxane, formamide and *tert.*-butanol. The activity attained under these conditions is 6% of the maximal value noted with emulsified tripropionin (12% of that with long-chain triglycerides). The effect is due to an increase of the V of the reaction, whereas K_m becomes less favorable. Some consequences of these findings on current concepts about the mechanism of lipase action are discussed.

MATERIALS AND METHODS

Organic compounds

Water was distilled in an all-quartz apparatus. The commercial compounds (Prolabo, France) were of the highest available standard. However, they were further purified in the laboratory before use. Dioxane was treated by solid KOH for 24 h, freed from peroxides by filtration through an activated Al_2O_3 column and distilled over metallic sodium. It was kept over sodium and under nitrogen, and used within 2 days. *Tert.*-butanol and acetonitrile were purified by known techniques [8, 9]. Formamide and glycerol were simply distilled under reduced pressure.

Purification of tripropionin. Solubility determinations

Commercial tripropionin (Fluka Switzerland; 15 g) was dissolved in 20 ml of an anhydrous hexane–ether mixture (90:10 v/v) and the solution was purified by passage through an Al_2O_3 column (2 cm × 18 cm). The oxide was previously activated by heating at 200 °C for 12 h and equilibrated with the above solvent mixture. Tripropionin was eluted by washing the column with the solvent and its purity was checked by thin-layer chromatography on silicagel G in an hexane–ether (50:50 v/v) system.

The solubility of pure tripropionin in water and in various organic compound–water systems was determined according to an already published technique [2]. It was found to be 2.8 mg/ml in water at 25 °C and to increase steadily with the concentration of the added organic solvent. For solvent concentrations of 7.5 and 15% by vol.* it attained, respectively, 3.4–4.0 with *tert*-butanol, 3.5–4.4 with acetonitrile, 4.0–5.2 with dioxane and 4.3–5.5 mg/ml with formamide.

Preparation of lipase

Lipase was purified according to the method of Verger et al. [10] from a defatted porcine pancreas powder. A single preparation was used throughout the assays. It contained the two isolipases L_A and L_B, and about 30% of the saturating

* Unless otherwise stated, the concentrations of the solvent in the solvent-water mixtures were given by vol.

amount of colipase [11]. Its specific activity was 3800, when measured at pH 9.0 and 25 °C with the aid of an emulsion of purified olive oil in arabic gum [12].

Activity measurements

The initial rates of tripropionin hydrolysis were measured with a recording Radiometer pH-stat at pH 7.0 and 25 °C under nitrogen. In most assays, the substrate consisted of 20 ml of a 2 mg/ml tripropionin solution in water or in an organic solvent–water mixture. The titrator was loaded with 20 mM NaOH. Spontaneous tripropionin hydrolysis was first recorded during a few minutes. Then, the desired amount of lipase was added and the recording was pursued for about 3 min. The enzyme activity was calculated from the difference. Full ionization at pH 7.0 of the propionic acid liberated in the course of the reaction was checked by titration of known samples of the pure acid under exactly the conditions prevailing during lipolysis. Stirring during the reaction was carefully adjusted to avoid the formation of air bubbles in the liquid phase.

Technique for checking the absence of micelles in the tripropionin solutions

The exclusive presence of monomeric tripropionin in the investigated mixtures was checked by taking advantage of the spectral perturbation induced in iodine by its inclusion into micelles [13]. This technique, known to be applicable to solvent–water mixtures [13], was first tested with the non-ionic detergent Triton X-100 in 7.5% acetonitrile at 25 °C. Under these conditions, the critical micelle concentration of the detergent was found to be 0.13 mg/ml.

The difference spectra of iodine for varying concentrations of the detergent are presented in Fig. 1. For concentrations higher than the critical value (Curve 1; 0.70 mg/ml), a broad peak with a maximum at about 370 nm is observed. This peak is still visible just above the critical micelle concentration (Curve 2; 0.18 mg/ml) and it disappears completely below this concentration (Curve 3; 0.07 mg/ml). The last three curves (4–6) show that 3 mg/ml tripropionin solutions in various solvent–water mixtures do not contain any detectable micelles. The same should be true, of course, for the 2 mg/ml solutions used throughout this work.

RESULTS

Effect of organic compounds on the hydrolysis of tripropionin monomers by lipase

Previous assays [1, 2] performed with water-soluble substrates (triacetin and tripropionin) at varying concentrations had already shown that the lipase activity on fully dispersed molecules in an aqueous system is very low. The curves presented in Fig. 2a confirm this finding. Lipase appears to be significantly active on 2 mg/ml tripropionin solutions in pure water. But, this activity is seen here not to exceed 0.3% of that observed when the same amount of enzyme is incubated with an excess of the emulsified triglyceride (0.6% of the activity towards long-chain triglycerides in the usual assay system). Considering that, for reasons discussed later, the above figures are probably overestimated, the formation of an interface in tripropionin solutions can be assumed to multiply the activity of pancreatic lipase at least 300-fold.

Another fact revealed by Fig. 2a is that this low activity on tripropionin monomers is considerably increased by the addition of one of the following water-miscible compounds to the system: dioxane, acetonitrile, formamide and *tert*-butanol. For the

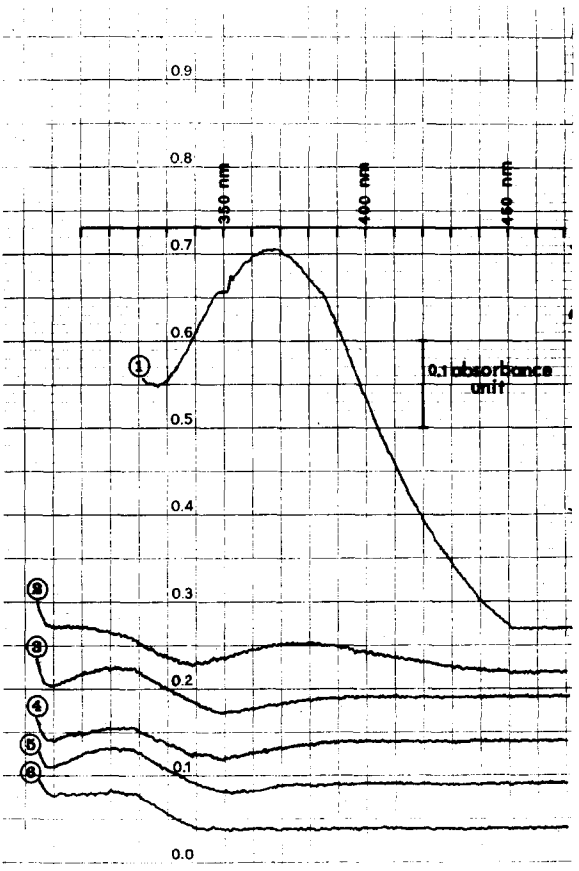

Fig. 1. Difference spectra of a 36 μg/ml iodine solution in the presence of Triton X-100 or tripropionin. Measurements were made with the aid of a Cary Spectrophotometer Model 14. Curves 1–3: 0.70, 0.18 and 0.07 mg/ml Triton X-100 solutions in 7.5% acetonitrile (critical micelle concentration of the detergent, 0.13 mg/ml). Curves 4–6: 3.0 mg/ml tripropionin solutions in, respectively, 7.5% acetonitrile, *tert*-butanol and dioxane.

first time, a system was found, in which lipase could display a substantial activity towards a monomeric substrate. In the particular assays illustrated by Fig. 2a, this activity is seen to attain 3.5–6% of the maximal value on emulsified tripropionin (7–12% of the value towards long-chain triglycerides).

A last remark concerning Fig. 2a is that the curves, except that related to glycerol (to be discussed later), are bell-shaped. At first, the lipolysis rate increases abruptly with the solvent concentration, then passes by a maximum for a solvent–water molar ratio of 1.5–2.7% (5–7.5%, by vol.) and finally decreases more or less sharply. This variation suggests the superposition of two opposite effects. It will be more easily interpreted in the following section.

Effect on the kinetic parameters of the reaction

Incubation assays in the absence of substrate indicated that the decrease of

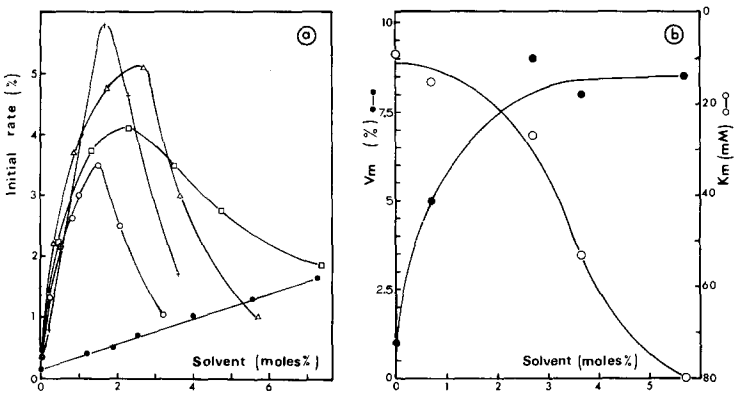

Fig. 2. Influence of the concentration of organic compounds on the lipase-catalyzed hydrolysis of tripropionin at pH 7.0 and 25 °C. In this figure, molar concentrations of the organic compounds are indicated. Rates are given in percent of the maximal rate observed with the same amount of lipase and an excess of a tripropionin emulsion, at pH 7.0 and 25 °C [2]. (a) Dependence of the initial rate of the reaction on the addition of dioxane (+), acetonitrile (△), formamide (□), *tert*-butanol (○) and glycerol (●). For each assay, 20 ml of a 2 mg/ml tripropionin solution were incubated with 4 units of lipase under pH-stat control. (b) Dependence of the kinetic parameters V and K_m of the reaction on the concentration of added acetonitrile.

lipase activity noted in the lower solvent concentration range was not due to enzyme instability. It could, therefore, be suspected that the rate dependence illustrated in Fig. 2a was related to a variation of the kinetic parameters V and K_m of the reaction. The corresponding assays were carried out as before, except for the substrate concentration that was modified according to needs. Linear Lineweaver–Burk reciprocal plots were obtained from which V and K_m values could easily be derived. For the assays performed in pure water, low enzyme activities were measured. Higher enzyme concentrations could not be used because the proportionality between enzyme concentration and reaction rate was lost at such concentrations. However, even in this case, the parameters could be evaluated with an acceptable accuracy.

The dependence of the parameters upon acetonitrile concentration is depicted in Fig. 2b. V is seen to increase sharply at first and to later reach an upper limit for a concentration of about 3 % (in moles). K_m also increases, at first slowly and then more abruptly. The obvious consequence is that the acceleration of the hydrolysis of tripropionin monomers by organic solvents is due to a rise of V. When the V curve flattens out, the unfavorable effect of the K_m increase becomes predominant and the initial rate drops. This subsequent drop, which depends on K_m, can be expected to be less important for higher substrate concentrations and even to disappear completely (as shown by the V curve in Fig. 2b) for saturating concentrations. In other words, the really significant phenomenon is the V increase occurring in the lower solvent concentration range, which probably corresponds to a more efficient catalysis under the newly created conditions.

Identification of the reaction products

The ability of lipase to hydrolyze monomeric tripropionin in organic solvent–water systems was confirmed by the finding that the triglyceride was completely converted into lower glycerides in the course of the reaction. The incubations in this case

were carried out at 25 °C and pH 7.0 under pH-stat control on 10 ml of the 2 mg/ml tripropionin solutions in 7.5% acetonitrile, dioxane and *tert*-butanol. Samples (50 μl) were removed when the overall hydrolysis attained 40% and they were submitted to thin-layer chromatography on silica gel plates as indicated in Fig. 3. This figure shows

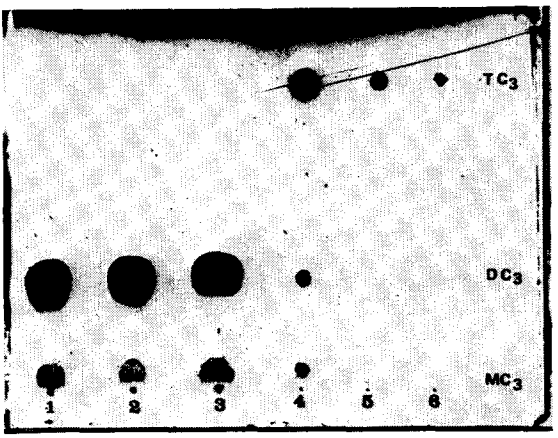

Fig. 3. Thin-layer chromatography on silica gel plates in an hexane–ether system (40:60, v/v) of 50-μl samples of tripropionin hydrolyzate in the presence of 7.5% acetonitrile (1), dioxane (2) and *tert*-butanol (3). Assay 4 was carried out with a reference mixture containing monopropionin (MC_3, 5 μg) + dipropionin (DC_3, 5 μg) + tripropionin (TC_3, 10 μg). Assays 5 and 6 were performed, respectively, with 5 and 2 μg of tripropionin. Free propionic acid arising from tripropionin hydrolysis was not stained by the reagent of Usui used for the detection of glycerides [14].

that tripropionin is no longer detectable in the hydrolysates (Assays 1–3), whereas the spot given by 2 μg of the triglyceride is clearly visible (Assay 6). It may be calculated that less than 2% of the original tripropionin remains after a 40% hydrolysis. For all practical purposes, therefore, tripropionin has been completely hydrolyzed.

Another point of interest shown by Fig. 3 is that dipropionin is very slowly degraded into monopropionin. Although the absence of free glycerol in the solutions was not checked, this observation suggests that lipase displays, in the homogeneous systems used here, the same "positional" specificity (exclusive hydrolysis of external chains in triglycerides) as that reported long ago for emulsified substrates [15–18].

DISCUSSION

Previous assays in this laboratory had shown that the addition of NaCl to aqueous solutions of tripropionin induced the formation of micelles [2]. A substantial increase of the activity of pancreatic lipase was simultaneously noted. This observation led to the assumption that the enzyme was able to act, not only on emulsified particles as already reported earlier, but also on much smaller micelles. All existing data, however, indicated that lipase activity on tripropionin monomers was very low in an aqueous system. This provided a convenient basis for the differentiation of lipases in general, for which a certain level of substrate aggregation was required, from ordinary esterases which appeared to act readily on monomers [1]. The addition of

low concentrations of various water-miscible organic compounds has now been shown to substantially increase the activity of pancreatic lipase on tripropionin monomers.

A first remark is that the figure of 0.3 % given above for expressing the activity of lipase on monomers in an aqueous system must be considered as a maximum, due to a possible activation of the enzyme by adsorption at glass surfaces [6]. If no activation occurred, however, the turnover of lipase on the monomers in aqueous solution would be $4 \cdot 10^3$ min^{-1}, a value not substantially different from those calculated for several ordinary esterases. In this perspective, the characteristic feature of lipase would not be to be weakly active on monomers, but to be considerably more active on emulsified particles, micelles and monomers in the presence of organic solvents.

It is clear, however, that the most important consequence of the new data presented in this report is that the existence of an interface or a certain state of aggregation of the substrate can no longer be considered as an absolute necessity for lipase to be substantially active. The data appear to be more consistent with the assumption that lipase activity, or more precisely, its catalytic power, is controlled by a parameter of water which would vary in the vicinity of an hydrophobic interface or, although with a much lesser efficiency, upon the addition of one of the organic compounds listed above. This variation could be assumed, either to affect the mechanism of the lipase-catalyzed reaction or, more likely, to induce a conformational change in the enzyme molecule [3]. This latter hypothesis has not yet been directly confirmed. But, it is supported by recent assays with monomolecular films of substrate (see ref. 19 for lipase and ref. 5 for phospholipase) and also by the existence of an essential carboxylate

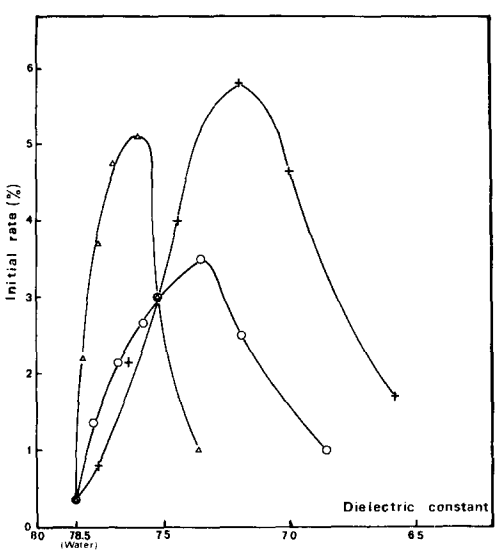

Fig. 4. Variation of lipase activity as a function of the dielectric constant of the organic solvent–water mixtures. The values of the constant at 25 °C of aqueous solutions of *tert*-butanol (○—○), acetonitrile (△—△) and dioxane (+—+) have been derived, respectively, from the data of Akerlof [23], Edsall [24] and Critchfield [25]. To our knowledge, similar data have not be published for formamide. Initial rates are given in percent of the maximal rate observed with the same amount of lipase and an excess of a tripropionin emulsion, at pH 7.0 and 25 °C [2].

in lipase reported to be involved in the stabilization of the active form rather than in the catalytic site proper [20].

The addition of water-miscible organic compounds to aqueous systems has already been shown to affect other enzymatic reactions and this influence has been attributed to a change in the dielectric constant of water [21]. If the same assumption was valid in the case of lipase, a single rate–dielectric constant dependence curve should in principle be obtained with all the compounds employed here. Fig. 4 shows, on the contrary, that the curves are distinct for three of these compounds (*tert*-butanol, acetonitrile and dioxane). The argument is still more convincing with formamide whose dielectric constant is higher than that of water (109 instead of 78.5 at 25 °C [22]) and which can, therefore, be expected to increase the constant rather than to decrease it for the same effect on lipase activity.

Another possibility is that the activity of lipase on dissolved tripropionin monomers is controlled by what is often designated as the "structure" of water [26], i.e. the degree and the mode of association of liquid water dipoles in the vicinity of the reacting molecules. This structure was repeatedly reported to be modified by the addition of low concentrations of the compounds enhancing lipase activity [27, 28], and also by the proximity of a solid or liquid interface [29]. Polyols like glycerol were observed to have an opposite effect and this may account for the widely different aspect of the corresponding curve in Fig. 2a.

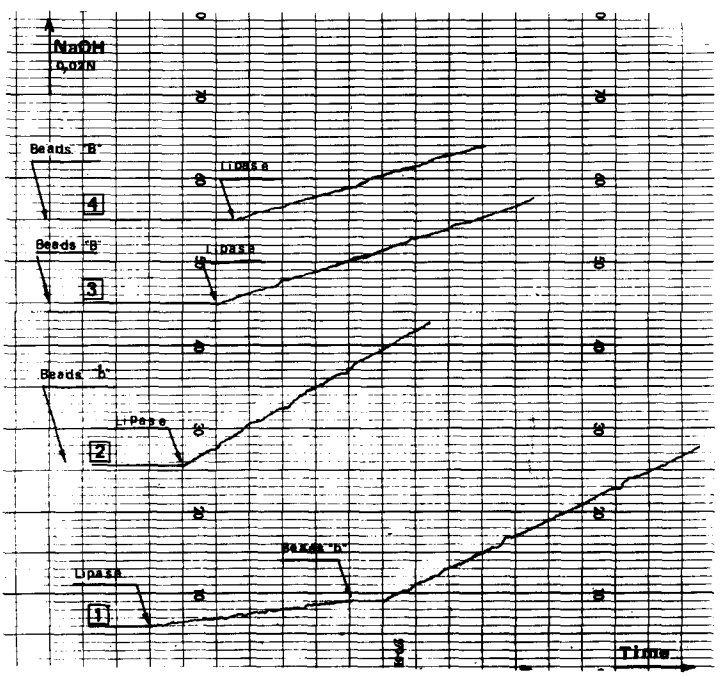

Fig. 5. Acceleration of tripropionin hydrolysis in water by addition of glass beads. Assays were conducted at 25 °C and pH 7.0 under pH-stat control with 20 ml of a 2.2 mg/ml aqueous solution of tripriopionin, 6 units of lipase and 500 mg of glass beads. The diameter of the beads was 0.5 mm for those designated "B" and between 0.1 and 0.2 mm for "b". The arrows indicate when lipase or beads were added to the tripropionin solution. The lines are original pH-stat recordings.

Finally, Fig. 5 shows that the addition of glass beads to a molecularly-dispersed tripropionin solution also induces a substantial acceleration of the lipase-catalyzed hydrolysis. A possible interpretation of this observation is that, in agreement with other authors' views [29], the solid interface of the beads modifies the structure of water in a way similar to that induced by organic solvents. But, enzyme activation may also be supposed in this case to result from adsorption at the glass surface [6]. Monophasic systems like those provided by solvent–water mixtures appear to open better perspectives towards a more precise understanding of the mechanism of lipase action.

As already pointed out in the Introduction, the possibility for the structure of water to affect lipase activity has already been considered before [4, 30]. But, so far, no experimental data had been given to support this interesting suggestion.

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